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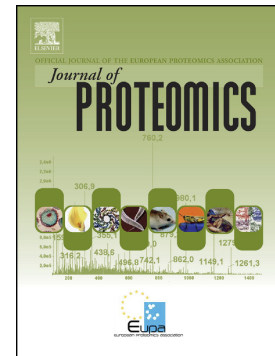
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Recombinant plant-derived human IgE glycoproteomics

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Abstract

The increasing biotechnological interest in human IgE antibodies demands advanced systems which allow their proper expression. However, this is still a challenge due to the complexity of the molecule, particularly regarding the diverse N-glycosylation pattern. Here, we present the expression of recombinant IgE in wild type and glycan-engineered *Nicotiana benthamiana* plants and in-depth N-glycosylation analyses. Mass spectrometric profiling revealed that plant IgE has a site occupancy rate that ranges from non-occupied at glycosite 6 (GS6) to 100 % occupancy at GS1 and 2. Similarly to human cell-derived IgE, plant versions carry complex N-glycans at GS1-5 and oligomannosidic structures at GS7. Computational modelling suggests that spatial position (or orientation) of glycans can impair processing or site occupancy on adjacent glycosites. IgE expressed in glycoengineered and wild type plants carry, respectively, GnGn and plant-typical GnGnXF structures at large homogeneity. This contrasts with the glycan diversity of HEK cell-derived IgE, carrying at least 20 different glycoforms. Importantly, IgE glycoengineering allows the control of its glycosylation, a so far unmet need when using well-established expression systems. This enables the elucidation of possible carbohydrate-dependent IgE functions.

Significance

Targeted glycosylation of recombinant proteins may provide an advantage in therapeutic applications. Despite increasing biotechnological interest in IgE antibodies, knowledge and impact of glycosylation on this antibody class are scarce. With the elucidation of the glycosylation profile and the ability to glyco-engineer recombinant IgE, we provide an important step towards the generation of IgE with other targeted N-glycans. This will facilitate detailed structure–function studies and may lead to the production of IgE with optimized activities.

1. Introduction

Due to recent breakthrough observations demonstrating glycosylation-dependent immunomodulatory effects of antibodies, glycobiology has become of special interest in immunology research. Immunoglobulin E (IgE), the least abundant immunoglobulin class in human serum, is principally known for its role in allergic responses [1], but it is also involved in anti-parasitic immune reactions [2]. In addition, although not yet fully understood, IgE may play an important role in the recognition of cancer by the immune system [3]. Recent studies also point to possible therapeutic applications of monoclonal IgE in the context of cancer [4, 5]. An outstanding feature of IgE antibodies is their complex glycosylation pattern: approximately 12 % of their molecular weight is carbohydrates, making them the most heavily glycosylated immunoglobulin class. This molecule contains seven potential N-glycosylation sites (GS1-7 at Asn21, 49, 99, 146, 252, 264 and 275; note that Asn are numbered from the start of the CH1 domain) in the heavy chain constant region (Fig. 1A). N-glycan profiling of serum IgE (from healthy and hyperimmune donors) revealed that GS7 (Asn275), located in the CH3 domain of the antibody, is exclusively decorated with oligomannosidic structures (OMS, Man5-Man9). While GS6 (Asn264) does not seem to be occupied, all other sites in the CH1-CH3 domains (GS1-5) carry complex-type N-glycans. The majority is bi-antennary, core-fucosylated structures with one or two terminal N-acetylneuraminic acid residues (70-80%); approximately 10-15 % of these N-glycans have bisecting GlcNAc [6, 7]. Notably, significant N-glycan diversity has been observed at each glycosite and between different (patho-)physiological stages [7-9]. While mounting evidence favours N-glycan involvement in the modulation of IgG activity, less is known about this important post-translational modification for other immunoglobulins, like IgE. Early studies have shown that at least precursor N-glycans (i.e. Glc3Man9GlcNAc2) are essential for the secretion of IgE [10]. The impact of fully processed (sialylated) N-glycans, which are the most abundant glycoform in human serum IgE, is still under debate. Researchers are particularly interested in understanding the interaction of IgE with effector cells of the immune system through their Fc receptors: the high-affinity FcεRI and the low-affinity CD23/FcεRII [11, 12]. Partially inconsistent results have been reported for FcεRI binding: while some studies concluded that IgE glycosylation is essential for FcεRI binding and pro-inflammatory effector functions [13-16], others did not find such correlation [17, 18]. A more recent study points to the requirement of an occupied C-terminal glycosite (carrying oligomannosidic N-glycans) for FcεRI binding and subsequent initiation of the IgE-mediated allergic cascade [19]. The IgE glycosylation status also seems to influence binding to FcεRII [20], an evolutionary-related form of the IgG receptor DC-SIGN. Nevertheless, more results are required to draw precise conclusions.

A major obstacle to elucidate N-glycan-dependent IgE activities is the current technological limitations to produce this antibody isotype with targeted N-glycan structures. *In vivo* glycoengineering is being developed in various expression systems as a method to control the composition of glycans and to enhance the pharmacological properties of recombinant proteins, including monoclonal antibodies [21-23]. Plant-based expression platforms have become a very attractive alternative to well-established expression systems, as the turnaround time from DNA to mg quantities of purified recombinant protein can be as short as one to two weeks [24]. An exciting aspect of using plants as production hosts is their ability for extensive glycoengineering (recently reviewed in [25]). Paradoxically, their limited endogenous glycosylation repertoire has turned out to be an advantage for generating proteins with homogeneous N-glycans, in contrast to the large glycome and the resulting N-glycan heterogeneity in mammalian cells, which prevents targeted manipulations. Importantly, wild type (WT) plants generate human-like complex N-glycans that most frequently terminate with GlcNAc residues (GnGn). However, they also synthesize N-glycan structures not present on mammalian proteins, i.e. core α 1,3-fucose and β 1,2-xylose residues (namely, GnGnXF). A central issue in N-glycan engineering is the generation of plants that lack plant-specific glycan residues [26]. Antibodies expressed in such mutant plants are decorated with GnGn structures, the mammalian core structure for further diversification (reviewed in [27]).

Despite the recently discovered potential of IgE as a therapeutic molecule, access to recombinant IgE is scarce. Recombinant expression of functionally active IgE has so far been reported in human and insect cells [4, 19, 28, 29]. However, data on their glycosylation status are incomplete. The expression of monoclonal IgE in HEK cells resulted in a large N-glycan diversity, with many new N-glycan structures in comparison to human serum IgE; on the other hand, insect cell-derived IgE exhibited an overall N-glycan pattern fundamentally different from a typical human serum IgE: instead of human type complex N-glycans (galactosylated and sialylated structures), they carried insect cell-typical structures that terminate with mannose residues [29]. Such an N-glycosylation pattern might have negative effects on the activity of therapeutic IgE. Although plants have proven to be a suitable host for the efficient expression and glycosylation of complex and functionally active human proteins (including antibodies like IgG, IgA and IgM) [27, 30], their applicability for the production of IgE has not been demonstrated yet.

Here, we focused on elucidating the N-glycosylation status of recombinant IgE antibodies produced in two different expression systems. An in-depth glycoproteomic analysis of monoclonal IgE targeted to growth factor receptor HER2/neu (namely, HER2-IgE), produced in mammalian cells (HEK293) and in *Nicotiana benthamiana* plants, was performed by liquid-

chromatography electrospray ionization-mass spectrometry (LC-ESI-MS/MS). We were able to obtain glycopeptides from IgE heavy chain covering all seven potential N-glycosylation sites by using a combination of three different proteases. Resulting spectra revealed information on site occupancy, site-specific glycosylation and overall glycosylation profiles.

2. Materials and methods

2.1. Cloning of HER2-IgE for plant based expression

MagnICON[®] vectors (courtesy of Icon Genetics, GmbH [31]) carrying the cDNA of HER2-IgE heavy and light chains [4] were generated in two steps: first, a 1287-bp cDNA fragment encoding the Ig epsilon chain constant region CH1-CH4 (UniProtKB - P01854) was codon-optimized and synthesized for *N. benthamiana* with flanking Bsmbl restriction sites (Invitrogen[™] GeneArt[™], <https://www.thermofisher.com>). To facilitate cloning of variable regions, Bsal sites were also introduced. The Bsmbl-Bsmbl fragment was cloned into the Bsal sites of the magnICON[®] TMV-based viral vector resulting in pICHα26211:IgEHc. Similarly, a 324-bp codon-optimized fragment containing the cDNA of the kappa chain constant region (UniProtKB - P01834) was amplified with primer pair LcFW/LcREV (Supplementary Table 1) that introduces Bsal restriction sites and flanks the fragment with Bsmbl restriction sites. The constant domain of the light chain was cloned into a PVX-based vector, resulting in pICHα31160:IgELc. In a second step, the Bsal restriction sites between the viral promoter and the constant region were used to insert the variable region. The cDNA encoding the variable domains of the light chain and heavy chain of trastuzumab (PDB ID: 1n8z [32]) were amplified with primers that introduce flanking Bsal restriction sites (LcHER2FW/LcHER2REV and HcHER2FW/HcHER2REV, respectively; Supplementary Table 1) and subsequently cloned into pICHα31160:IgELc and pICHα26211:IgEHc plasmids, resulting in the plant expression vector pICHα31160:HER2-IgELC (or PVXαHER2-IgELC) and pICHα26211:HER2-IgEHC (or TMVαHER2-IgEHC) (Supplementary Fig. 1).

2.2. Expression and purification of HER2-IgE

Nicotiana benthamiana WT and ΔXT/FT plants [26] were grown in a growth chamber at 22°C with a 16 h light/ 8h dark photoperiod. Leaves of 4–5 week old plants were used for agro-infiltration. Agrobacteria (strain GV3101 pMP90) transformed with either PVXαHER2-IgELC or TMVαHER2-IgEHC vectors were grown in liquid culture at 29 °C for 24 h, harvested by gentle centrifugation (5 min at 3000 g) and resuspended in infiltration buffer (10 mM MES pH 5.6; 10 mM MgSO₄) as previously described [33]. The two recombinant bacteria strains were mixed and

diluted to a final OD₆₀₀ of 0.1 for infiltration into WT (^{WT}HER2-IgE) and Δ XT/FT (^{Δ XF}HER2-IgE) plant leaves. 4-5 days after infiltration, leaves were harvested and flash-frozen in liquid nitrogen. Total soluble proteins were extracted in 1.5 M NaCl, 45 mM Tris, 1 mM EDTA, and 40 mM ascorbic acid (2:1 buffer/fresh weight) and recombinant IgE was purified by affinity chromatography (Protein A SepharoseTM Fast Flow, GE Healthcare). ^{WT}HER2-IgE and ^{Δ XF}HER2-IgE were eluted with 0.1 M Glycine/HCl (pH 2.7), neutralized with 1 M Tris (pH 9) and dialysed against PBS.

Cloning and expression of HER2-IgE in HEK293 cells (^{HEK}HER2-IgE) was described earlier [28]. Briefly, the heavy and light chains of HER2-IgE were cloned in pViro-2-hygro-mcs (InvivoGen) using the Polymerase Incomplete Primer Extension (PIPE) cloning method. Subsequently, HER2-IgE was transiently expressed in Expi293TM cells (Thermo Fischer Scientific) using the ExpiFectamineTM 293 transfection kit (Thermo Fischer Scientific), according to the manufacturer's instructions. ^{HEK}HER2-IgE was purified using a HiTrap KappaSelect (GE Healthcare) pre-packed column. Antibodies were eluted with 0.2 M Glycine/HCl (pH 2.3), neutralized with 1 M Tris (pH 9) and dialysed against PBS. Serum IgE (^{serum}IgE, obtained from a polysensitised allergic patient after informed consent was given) was purified by affinity chromatography using sepharose-coupled anti-IgE monoclonal antibody [34]. IgE was eluted using 5 M MgCl₂ and subsequently exchanged for PBS with Amicon Ultra Centrifugal filter tubes (Merck Millipore, Darmstadt, Germany). Purified IgE samples were reduced with 2-mercaptoethanol at 70 °C for 5 min and analysed by SDS-PAGE on a 12 % gel that was subsequently stained with Coomassie Brilliant Blue R 250 (Carl Roth GmbH + Co. KG).

2.3. Glycopeptide mass spectrometry analysis

SDS-PAGE protein bands corresponding to the heavy chain (at ~75 kDa) were excised and S-alkylated with iodoacetamide. Subsequently, the samples were proteolytically digested in 25 mM NH₄HCO₃ with either trypsin (Promega), proteinase K (Sigma Aldrich) or a combination of trypsin and chymotrypsin (Roche). This procedure allowed the extraction of glycopeptides covering all seven glycosites: GS1: NIPSNATSVTL; GS2: DTGSLNGTTM; GS3: VAHTPSSTDWVDNK; GS4: TINIT; GS5: GTVNLTW; GS6: ASGKPVNHSTR, and GS7: NGTLTVTSTLPVGTR. GS1 and 2 were obtained by the combined trypsin and chymotrypsin digestion; GS3, 5, 6 and 7 are tryptic peptides, and GS4 was obtained by digestion with proteinase K. After digestion, the peptides were extracted by adding 50 μ L 25 mM NH₄HCO₃ and then 50 μ L 100% ACN with two additional washes with 50 μ L 5% formic acid. The pooled extracts were dried in a centrifugal vacuum concentrator. The peptide mixture was analysed using a Dionex Ultimate 3000 system

directly linked to a Q-TOF instrument (maXis 4G ETD, Bruker) equipped with the standard ESI source (end plate offset 500 V; capillary 4500 V; dry gas (nitrogen) 5.0 L/min; dry temp 200°C) in the positive ion, data dependent acquisition mode. MS-scans were recorded (range: 150-2200 m/z, spectra rate: 0.5 Hz). Instrument calibration was performed using an ESI calibration mixture (Agilent). For peptide separation, a Thermo BioBasic C18 separation column (5 µm particle size, 150 x 0.32 mm) was used, with the solvent system recently described [30]. For the relative quantification of the different glycoforms, peak areas of EIC (Extracted Ion Chromatograms) of the first four isotopic peaks were summed. All observed charge states and adducts (ammonium), as well as the formation of formylated glycopeptides, were considered. MS/MS spectra were used for the verification of the glycopeptides by detection of oxonium ions HexNAc ($m/z = 204.1$), Hex+HexNAc ($m/z = 366.1$) and the unique Y1 ion (peptide+HexNAc). N-glycosylation site occupancy was deduced from the ratio of deamidated to unmodified peptide upon N-glycan release with 0.15 mU of PNGase A (Europa Bioproducts) overnight at 37 °C. For serum IgE, due to technical reasons, the summed peak areas of all glycoforms were compared to the area of the unglycosylated peptide. This method gave very similar values when compared to the standard approach

2.4. Molecular Modelling

A molecular model of human IgE-Fc was produced using both the bent (PDB ID 1O0V [35]) and extended (PDB ID 4J4P [36]) crystal structures of IgE-Fc. As representative of complex N-glycans, we modelled GnGn structures ($\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$) in GS4 and 5; and as representative of OMS in GS7, Man5 structures ($\text{Man}_5\text{GlcNAc}_2$) were modelled. Glycan structures were modelled using the glycan fragment database [37]. The energy was minimized to relieve steric clashes using the molecular operating environment (MOE; Chemical Computing Group, Inc. 2014) and the Amber99 force field. For GS5, additional GnGn structures were generated in the extended model of IgE-Fc to show that the N-glycan is free to take a broad range of conformations.

3. Results

3.1. Expression of monoclonal IgE in mammalian cells and *Nicotiana benthamiana* plants

For recombinant IgE expression, we used a monoclonal antibody targeted to the growth factor receptor HER2/neu (namely, HER2-IgE [4]). This antibody variant is analogous to the clinically approved IgG1 trastuzumab, the standard of care in HER2-overexpressing breast cancer. The heavy and the light chain of HER2-IgE were cloned into viral-based expression vectors

(PVX α HER2-IgELC, TMV α HER2-IgEHC) and transiently expressed in *Nicotiana benthamiana* wild type (WT) and the glycosylation mutant plants Δ XT/FT, which synthesize human type bi-antennary complex N-glycans lacking plant-specific xylose and core fucose residues [26]. Plant leaves were harvested 4-5 days post-infiltration and IgE was purified by immune-affinity (WT HER2-IgE, $^{\Delta XF}$ HER2-IgE). Similarly, appropriate HER2-IgE constructs were transiently expressed in HEK293 cells and immune-affinity purified (HEK HER2-IgE). As control, human serum IgE (serum IgE) obtained from a polysensitised allergic donor was included in our analyses. SDS-PAGE analysis of the four samples under reducing conditions exhibited two major bands corresponding to the size of the IgE heavy and light chains (75 and 25 kDa, respectively) (Fig. 1B). Compared to the human versions, plant-derived WT HER2-IgE and $^{\Delta XF}$ HER2-IgE exhibit a slightly reduced molecular mass of the heavy chain, most probably due to differences in glycosylation. In some samples, a weak signal at 55 kDa appeared, corresponding to a degradation product of the heavy chain. Importantly, SDS-PAGE analysis of plant-derived HER2-IgE under non reducing conditions indicates correct assembly into heterodimers (Supplementary Fig. 2).

3.2. Glycosite occupancy

IgE heavy chains carry three glycosites on the CH1 domain (GS1-3), one on the CH2 domain (GS4) and three on the CH3 domain (GS5-7) (Fig. 1A). In order to analyse all seven glycopeptides, the IgE heavy chains were proteolytically digested with different enzymes. GS1 and 2 were obtained by combination of trypsin and chymotrypsin digestion; GS3, 5, 6 and 7 are tryptic peptides, and GS4 was obtained by digestion with proteinase K. Apart from our limitation of not finding GS4 upon proteinase K digestion, we find occupancy rates for serum IgE similar to those previously reported by Plomp *et al.* [7]. Remarkably, the overall pattern of site occupancy was similar for all four samples tested (WT HER2-IgE, $^{\Delta XF}$ HER2-IgE, HEK HER2-IgE, serum IgE) (Fig. 2). The degree of occupation of GS1 and 2 was found to be virtually 100 % in all four samples. Differences between plant- and human-derived IgE site occupancies were observed for GS3 and 5. While these sites are efficiently occupied in human cell-derived variants (75-90 %), decreased levels were detected in the plant versions (18-48 %). Just as previously reported for human serum IgE [7], no IgE variant was glycosylated at GS6 (Fig. 2 and Supplementary Table 2).

3.3. Site specific N-glycan profiling

The glycosylation status of the different IgEs was analysed regarding the relative abundance of different glycoforms at each glycosite by LC-ESI-MS/MS (Fig. 3 and Supplementary Tables 3

and 4). The MS spectra of all four IgE samples exhibited the presence of complex N-glycans at GS1-5, oligomannosidic structures (OMS) at GS7 and unglycosylated GS6 (Fig. 3 and Supplementary Tables 3 and 4). Complex glycosylation of plant-derived IgE is relatively homogenous in both WT and Δ XT/FT plants. GS1-5 showed a single dominant glycoform, namely GnGnXF or GnGn, for WT plants (WT HER2-IgE) and Δ XT/FT plants ($^{\Delta XF}$ HER2-IgE), respectively. This glycoform accounts for up to 75 % (Fig. 3 and Supplementary Table 3). In addition, some OMS appeared (up to 27 %) and $^{\Delta XF}$ HER2-IgE also carried traces of fucosylated structures (GnGnF, up to 11.5 %), most probably due to incomplete RNAi-based downregulation of α 1,3-fucosyltransferase expression [26].

Four glycan species were detected at GS1-5 of serum IgE: mono- and bi-sialylated bi-antennary N-glycans with core fucose (ANaF and NaNaF), and bisected structures (ANaFbi and NaNaFbi). Notably, our studies do not allow the discrimination of bisected bi-antennary structures from tri-antennary structures (e.g. NaNaFbi from GnNaNaF); however, due to previous results obtained from serum IgE analysis [7, 9], we anticipate the presence of bisected glycoforms. These bisected structures dramatically increase at GS5 (up to 76 %) (Fig. 2 and Supplementary Table 3). In contrast to serum IgE, in HEK HER2-IgE the glycosylation of GS1-5 is extremely heterogeneous (Fig. 2 and Supplementary Table 4). In GS1-4, up to 20 different glycoforms were detected. They mainly consist of galactosylated tri- and tetra-antennary N-glycans with 1-4 sialic acid residues. N-glycans at GS5 differ from GS1-4 in that they generally consist of bisected bi-antennary structures terminating with GlcNAc, galactose or sialic acid residues. No branched structures were detected on that site. Overall, the glycan diversity in HEK HER2-IgE is significantly increased in comparison to the plant- and serum-derived IgEs.

Regarding GS7, all four IgE samples carry almost exclusively oligomannosidic structures, ranging from Man5 to Man9. While Man5-7 are the most abundant glycoforms in HEK HER2-IgE and serum IgE, in plants we observe an even distribution of Man5-9. Surprisingly, GS7 of plant-derived HER2-IgE is also decorated with hybrid N-glycans, Man5Gn and Man4Gn (up to 20 %).

3.4. IgE Modelling

To assess a possible impact of the structural conformation of human IgE on its glycosylation, a molecular model based on previously published Fc structural information was built. IgE-Fc, which harbours GS4-7, was crystallographically observed in an extended and a bent structure (Fig. 4) [35, 36]. In the bent structure (Fig. 4B), which has computationally been identified as the energetically favoured one (PDB ID: 4J4P [36]), the CH2 domain pair folds back onto the CH3-CH4 domains, both in free and complex forms (PDB IDs: 1O0V [35] and 2QWR [38]).

Glycosylation modelling of GS4, 5 and 7 suggests that further processing of the OMS at GS7 may be sterically hampered by the CH2 domains in the bent conformation (Fig. 4B). Considering the distance at the amino acid sequence level, GS6 is in close proximity to GS5 and GS7 (12 and 11 amino acids apart, respectively). In the 3D structure, GS5 and GS6 are on the same beta strand, which confers them relatively close physical proximity. By contrast, GS6 and GS7 are oriented in different directions (Fig. 4B). These spatial orientations might have an impact on glycan accessibility and processing. Moreover, the potential conformational diversity of complex N-glycans at GS5 (visualized by a superposition of several GnGn structures on Fig. 4A) suggests that GS5 glycans are on the surface and could interfere with the glycosylation of GS6.

4. Discussion

Despite the increasing knowledge about the important role of IgE antibodies in various immunological processes, their low serum concentration renders them the least characterized antibody isotype. Their powerful physiological role in allergic sensitization in combination with their potential therapeutic use has intensified research interest in human IgE antibodies with tailored specificities. However, due to the complexity of the molecule, recombinant expression is challenging and has mainly been restricted to mammalian cells. While such cells seem to be well suited for the expression of functionally active IgE [4, 19, 28], their usability for targeted glycosylation is hindered by the large glycan diversity in mammalian cells. Here, we pursued the expression of recombinant IgE in plants with a special focus on its glycosylation status in comparison with a HEK cell-derived version. We aimed to assess the use of plants for the production of IgE with a targeted glycosylation profile. ~~elucidation of the glycosylation status of recombinant IgE produced in human cells and plants, in comparison with human serum-derived polyclonal IgE.~~ As a model, we used anti-HER2/neu IgE that retained the functions of its IgG counterpart (trastuzumab) on restricting proliferation of HER2/neu-expressing tumour cells and activating effector cells to kill tumour cells (HER2-IgE) [4].

We found that the method developed in [7] proves to be a valuable tool for in-depth glycoproteomic analysis of monoclonal HER2-IgE. We showed that the type of glycans (complex or oligomannosidic) present at each N-glycosylation site of HER2-IgE is consistent with previous studies using serum IgE [7, 9]; namely, GS7 at the CH3 domain exhibits OMS, while complex glycans are found on GS1-5. Albeit known from other proteins, including IgM and IgD [39], the presence of complex and oligomannosidic N-glycans on a single protein is a peculiarity. To date, the biological relevance, as well as the processes that drive this differential glycan processing, are poorly understood. One model refers to different accessibility of the glycosylation sites either by glycosylation from adjacent glycosites (IgD [6]) or as a consequence of protein

oligomerization (IgM [40]). For IgE, it was proposed that access of OMS to glycosylation-processing enzymes is blocked by the CH2 domain [6]. Molecular modelling suggests that, indeed, processing of the OMS towards complex structures at GS7 may be sterically hampered by the CH2 domain, at least in the bent conformation. Moreover, our modelling studies imply inaccessibility of GS6 due to sterical hindrance of complex glycans present on the adjacent GS5. Our studies suggest that such structures can take a broad range of conformations, influencing the properties of the environment. Further studies are required to confirm these findings. According to sequence alignments, it was suggested that GS7 of IgE is the equivalent of the single IgG glycosite (Asn297) and Asn402 of IgM antibodies [7]. Notably, the IgG-Fc glycosite is occupied by complex-type N-glycans, and the impact of the glycan composition on binding to FcγR is well known [41]. Glycosites with OMS in IgE and IgM are located on the CH3 domain, surrounding the proposed Fc receptor binding sites. This suggests that they may have critical impact on the ability of the antibody to bind to the corresponding Fc receptors and to subsequently induce downstream (effector) functions. Indeed, one study refers to the absolute requirement of a glycosylated GS7 for IgE binding to the FcεRI and subsequent downstream activities [19]. Still, to what extent particular OMS impact this activity is not known. Unexpectedly, significant differences were found when comparing the composition of complex N-glycans of serum- and HEK cell-derived IgE. The most striking alteration was the presence of large amounts of galactosylated tri- and tetra-antennary N-glycans carrying 1-4 sialic acid residues in ^{HEK}HER2-IgE. As a consequence, the number of glycoforms increased fivefold in ^{HEK}HER2-IgE compared to ^{serum}IgE. A similar observation was made by Shade *et al.* [19], who also expressed a monoclonal IgE in HEK cells. The differences in glycosylation between ^{HEK}HER2-IgE and ^{serum}IgE should be interpreted with caution, as the latter is naturally polyclonal and it is derived from a single (allergic) donor. Notably, we also observed the absence of glycans at GS6 on ^{HEK}HER2-IgE. This is in accordance with serum- derived IgE, however contradicts previous results reporting full occupancy of this site on HEK cell-derived monoclonal IgE [19]. Interestingly, in plant-derived IgE (^{WT}HER2-IgE and ^{ΔXF}HER2-IgE), complex N-glycan profiles of GS1-5 are very similar. However, for ^{HEK}HER2-IgE and ^{serum}IgE, distinct complex N-glycans are observed in GS5 (located at the CH3 domain), compared to the similar profiles on GS1-4 (located at CH1 and CH2 domains). This was also previously observed for healthy serum IgE [7]. Little is known about HEK cell-specific glycosylation. Monoclonal IgG produced in such cells exhibit a similar glycosylation profile as serum IgG, with AAF, AGnF and GnGnF being the three major structures (i.e. G2F, G1F and G0F [42]). An exception is the presence of unusually high amounts of bisecting structures in some variants (up to 40 % [43]).

At this moment, it cannot be ruled out that the observed HEK cell-derived glycosylation is restricted to our monoclonal HER2-IgE antibody, although it could also be a general characteristic of the expression system. The fact that another recombinant IgE antibody with a different specificity expressed in HEK cells also carries a large amount of branched structures (with and without sialic acids) [19] points to a peculiarity of HEK cells. Yet, more data on HEK cell-derived recombinant proteins is needed before drawing general statements on the glycosylation pattern of these cells.

Essentially, we show that plants are able to express IgE and that its glycosylation status is similar to human cell-derived IgE, regarding both N-glycan type and site occupancy. In plants, GS1-5 are occupied by complex type N-glycans, GS6 is not occupied and OMS are detected in GS7. Some differences in the level of glycosylation occupancies are observed for GS3 and 5. At the moment, we cannot explain the decreased site occupancy in plant-derived samples, although this has also been observed for other recombinant plant-produced proteins [44]. Overall, it seems that the general molecular mechanisms of plant and mammalian N-glycosylation processing are highly conserved, an unexpected fact given the large phylogenetic distance between the two phyla.

Importantly, we were able to generate a glycan engineered HER2-IgE in plants that is decorated with GnGn structures which feature large homogeneity (Δ^{XF} HER2-IgE). This common eukaryotic core glycoform can be used for further targeted diversification towards glycans usually found on serum IgE, or towards novel structures, to possibly modify or even enhance the activities of the antibody. Using the recently developed plant-based expression system that relies on the versatile combination of glycan engineered plants with transient expression modules [25], it should be possible to generate IgE antibodies with additional targeted glycosylation profiles and to subsequently trace their functionalities. This approach will allow the reconstruction of complex glycoforms, including sialylation, at great homogeneity [45], a so far unmet need for elucidation of glycan-dependent activities.

5. Conclusion

Although HEK cell -derived and human serum IgE have common glycosylation features, there are unexpected differences. These variations need to be considered when using human cell lines for the expression of recombinant proteins. Plant-based IgE expression points to the ability for targeted glycosylation, which may open new ways for glycan-dependent IgE structure-function studies. This will not only help to better understand basic IgE-based mechanisms on the regulation of the immune system, but may also be used for the development of antibody-based therapies, an emerging field in drug development.

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Figure captions

Fig. 1. (A) Schematic representation of the structure and glycosylation status of human serum IgE. Monomers of IgE consist of two heavy chains (ϵ chain) and two light chains. The ϵ chain contains 4 immunoglobulin-like constant domains (CH1-CH4) and one variable domain (VH), while the light chain contains one constant (CL) and one variable domain (VL). Glycosites (GS) 1-7 on the ϵ chain represent Asn21, 49, 99, 146, 252, 264 and 275. (B) Coomassie brilliant blue stained SDS-PAGE (under reducing conditions) of purified IgE antibodies (~500 ng). IgE samples are derived from (1) *Nicotiana benthamiana* wild type plants (WT HER2-IgE); (2) *Nicotiana benthamiana* glycosylation mutant Δ XT/FT ($^{\Delta XF}$ HER2-IgE); (3) HEK293 cells (HEK HER2-IgE) and (4) human serum (serum IgE, single donor with a hyperimmune condition). HC, LC: heavy and light chain, respectively. (M) Molecular weight indicated in kDa.

Fig. 2. Site-specific glycosylation occupancy (%). IgE expressed in *Nicotiana benthamiana* wild type plants (WT HER2-IgE), glycosylation mutant Δ XT/FT ($^{\Delta XF}$ HER2-IgE), HEK293 cells (HEK HER2-IgE) and human serum (serum IgE) was monitored. Values were obtained using two different methods (see Materials and Methods and Supplementary Table 2). Glycosylation efficiency on GS4 in serum IgE was not determined, as the glycopeptide resulting from Proteinase K digestion was not found.

Fig. 3: LC-ESI-MS/MS analyses. Relative abundance of glycoforms of IgE expressed in plants (WT HER2-IgE, $^{\Delta XTFT}$ HER2-IgE) and in human cells (serum IgE, HEK HER2-IgE). Schematic representations of N-glycans detected by mass spectrometry at each glycopeptide (GS) are shown (symbol nomenclature in accordance with the Consortium of Functional Glycomics). Note that for HEK HER2-IgE more than 20 glycoforms were found and thus were grouped in this figure as explained in Supplementary Table 4. Glycoforms present in less than 5% were group in "Others". For detailed information see Supplementary Tables 3 and 4.

Fig. 4: Molecular model of IgE-Fc. The model contains (CH2, CH3 and CH4 domains) based on the extended and bent crystal structures. The protein backbone is coloured in red shades (Chain A) and blue shades (Chain B) with CH2-CH4 domains coloured from dark to light shades. (A) Extended structure. A wide range of possible GnGn structures is modelled on GS5 in Chain A (red arrow) to exhibit the glycan flexibility and proximity to the unoccupied GS6 (black spheres). (B) Bent structure. The accessibility to the OMS on GS7 (green) is hindered due to the folding of the CH2 domains onto the CH3 and CH4 domains. GS: glycosite. Glycan modelling was performed according to the glycan fragment database [37].

Figure 1

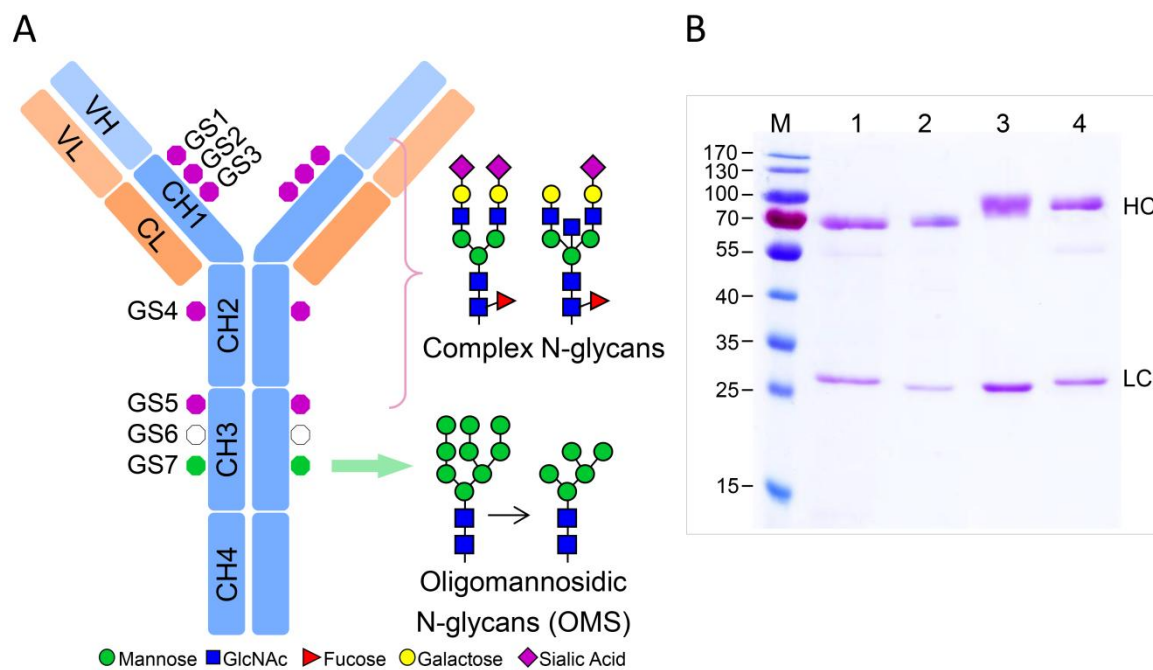


Figure 2

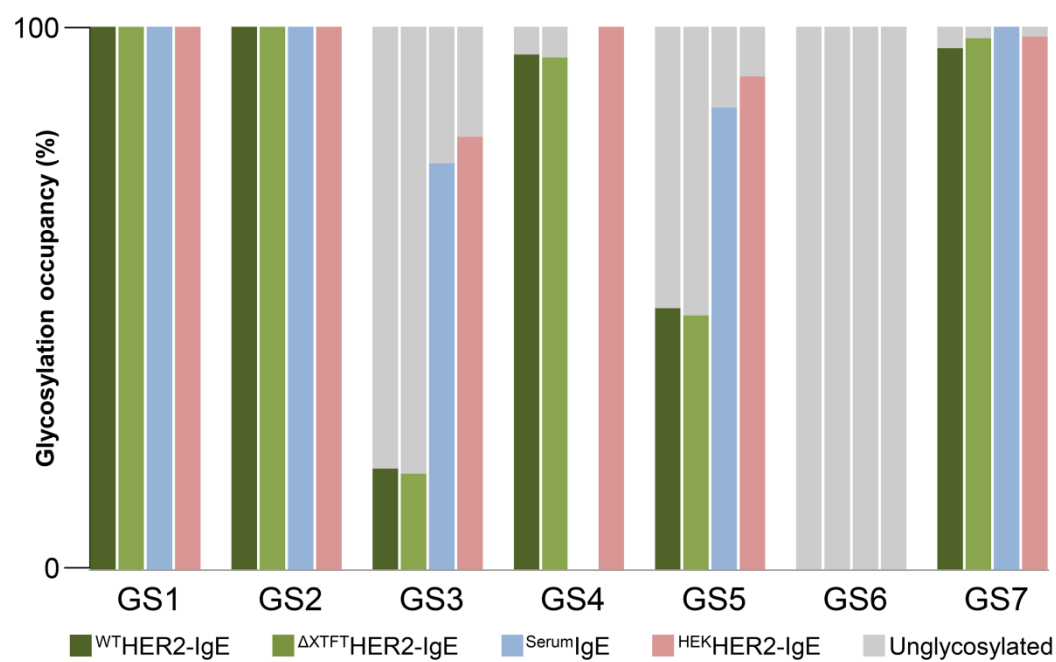


Figure 3

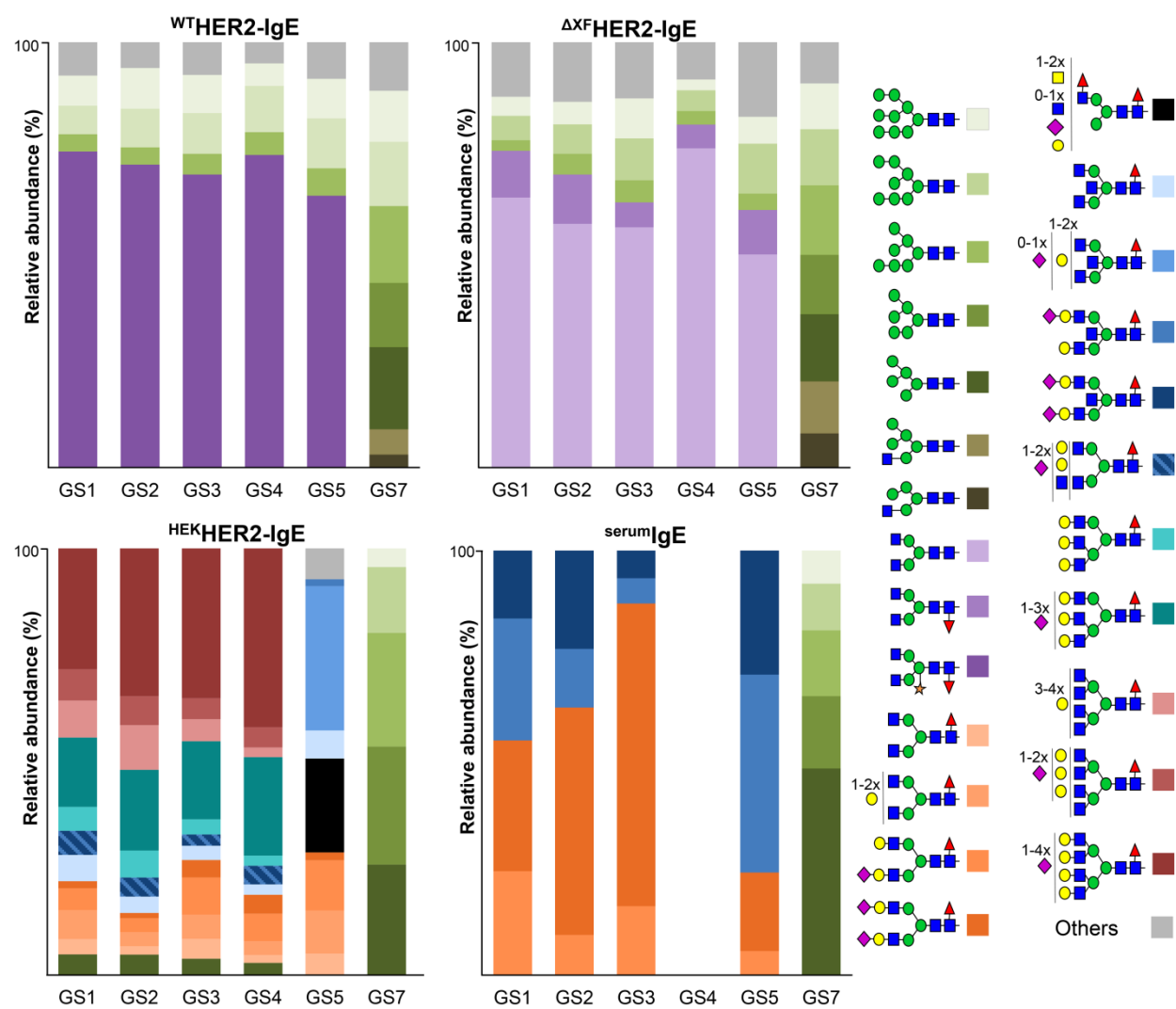
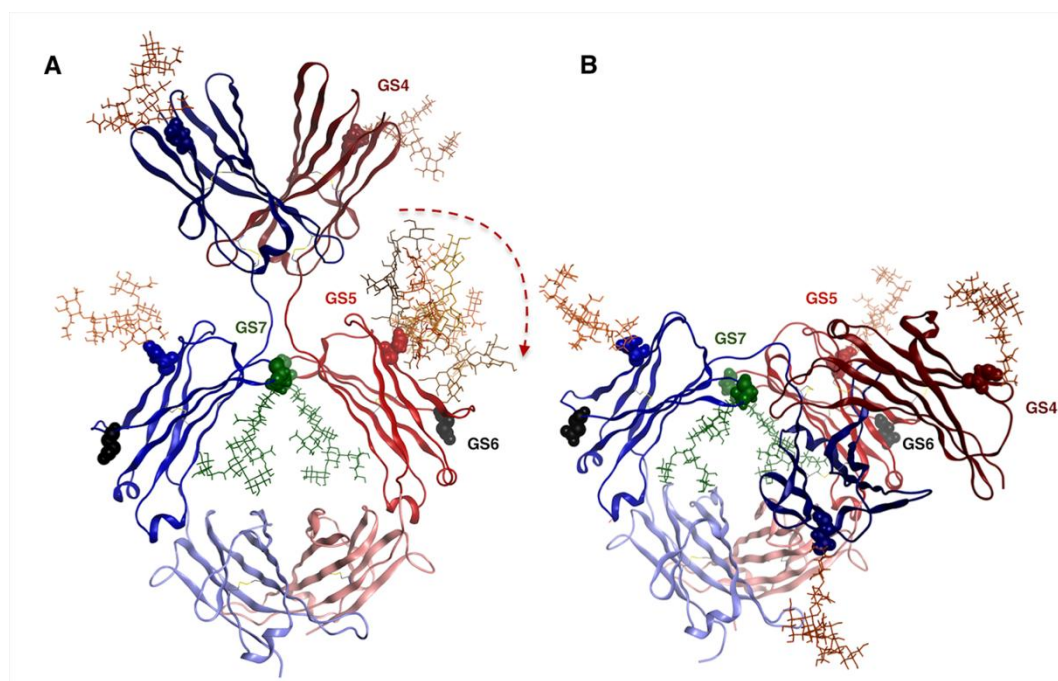


Figure 4

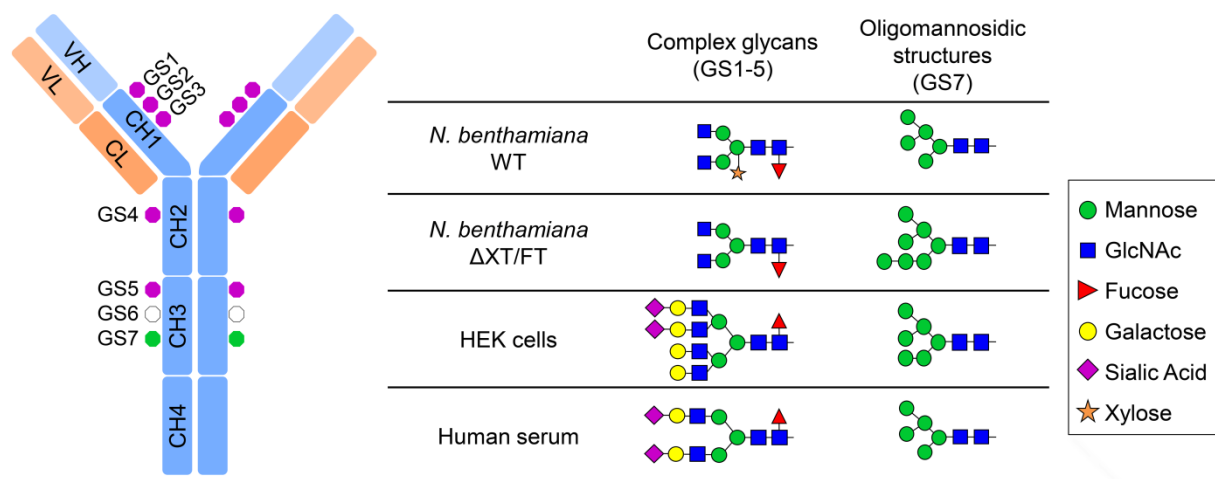


Conflict of interest

The authors declare no conflict of interest.

ACCEPTED MANUSCRIPT

Graphical abstract



“Highlights”

- IgE glycosylation can be engineered towards targeted structures
- HEK cell- and serum-derived IgE differ in their glycosylation pattern
- Molecular modeling suggests sterical hindrance as an important factor in N-glycan processing

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